Peer Review File

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Reviewer A

The authors investigated disulfidptosis as a new type of cellular death triggered in response to disulfide stress in hepatocellular carcinoma (HCC). This study is pretty bioinformatic but provides a clear methodology and data that can be useful for the oncologist. I would strongly add a table on how disulfidptosis differentiates from other forms of cell deaths and an expansion of the limitations of the study, which is not conclusive at this stage.

Reply: Thank you very much for your comments. Cell death is a very complex process, and there are many ways in which cells die, including apoptosis, ferroptosis, necroptosis, and cuprotosis, etc. disulfidptosis is very different from them. What's more, there are limitations of our study. Therefore, we have added relevant content in the discussion section. (see Page 14, line 452-454)

Changes in the text: Third, beyond disulfidptosis, other cell death mechanisms, including apoptosis, ferroptosis, necroptosis, and cuprotosis, are intricately linked to liver cancer and warrant further investigation.

Reviewer B

This paper has done a comprehensive study using the machine learning method and Cox-type survival analysis in disulfidptosis-related genes of prognostic signature and immune infiltration features in HCC. Overall, the analysis procedure is typical in medical prognosis studies, which is fine, although it can be improved.

Reply: We greatly appreciate your comments. This study aims to explore the relationship between disulfur-related mortality and hepatocellular carcinoma (HCC). The study also seeks to construct the prognostic features of disulfidptosis-related genes (DRGs) in HCC using relevant bioinformatics methods. Our findings indicate that a model incorporating the DRGs signature can accurately predict HCC patient prognosis. We evaluated the predictive utility of relevant clinical features, their role in the immune context, and their potential in immunotherapy. Additionally, we verified the expression of the DRGs signature in HCC through cell experiments. The DRGs signature is anticipated to be a novel biomarker with promising applications in prognosis evaluation, immunotherapy, and drug sensitivity prediction for HCC patients.

Major comments:

Looking at Figure 1 Flow Chart, it's not clear to me what has been done in the analysis. At the top level, there are 24 disulfidptosis genes, then at the next level, the number decreased to 16, and then to 6. Such a procedure can cause a selection bias. Then, the problem came in using LASSO. I don't understand why the authors need LASSO as the total number of genes is just 6. Four genes were selected, and the analyses were based on these four genes. There are questions remaining: Are those 24 disulfidptosis genes representative, i.e., have any other genes been missed?

Reply: Thank you very much for your comments. Differential analysis was conducted on liver cancer sequencing data from the TCGA and GEO databases to identify differentially expressed genes. These were then cross-referenced with 24 DRGs, yielding 16 DRGs with differential expression. To screen for genes with prognostic relevance, a differential analysis of the 16 identified genes was performed, resulting in 6 genes showing significant differences in expression and survival prognosis. This genetic screening method is generally practical (1,2,3). The Least Absolute Shrinkage and Selection Operator (LASSO) is a data mining technique that incorporates a penalty function into traditional multiple linear regression. This function continuously compresses coefficients, simplifying the model to prevent collinearity and overfitting. This method also achieves variable selection when coefficients are reduced to zero. Consequently, LASSO regression offers an efficient solution for variable selection. Unlike traditional stepwise regression, which selects variables in a forward and backward manner, LASSO can handle smaller sample sizes and screen a larger number of variables more effectively. For instance, LASSO regression is widely applicable in areas such as genomics, imaging, and other fields involving small sample analysis. To circumvent collinearity and overfitting, and to identify genes for a more parsimonious model, LASSO analysis was employed for the further examination of the 6 identified genes.

On February 6, 2023, the MD Anderson Cancer Center published research in Nature Cell Biology, revealing the mechanism of disulfide stress-induced cell death and introducing the term "Disulfidptosis" for this new cell death type (4). The study demonstrated disulfoxide's role in gene and death regulation, using a gene set that is considered relatively representative. However, research on DRGs is continuously evolving, and we have incorporated as many other DRGs as possible. This study highlights disulfoxide's role in gene regulation and cell death. The gene set used in this research is considered to be relatively representative. However, as research on the disulfur death gene set is continuously updated, we have included as many additional disulfur death genes as possible.

References:

1. Chen Y, Tang L, Huang W, Abisola FH, Zhang Y, Zhang G, Yao L. Identification of a prognostic cuproptosis-related signature in hepatocellular carcinoma. Biol Direct. 2023 Feb 7;18(1):4. doi: 10.1186/s13062-023-00358-w. PMID: 36750831; PMCID: PMC9903524.

2. Cheng Z, Chen Y, Huang H. Identification and Validation of a Novel Prognostic Signature Based on Ferroptosis-Related Genes in Ovarian Cancer. Vaccines (Basel). 2023 Jan 17;11(2):205. doi: 10.3390/vaccines11020205. PMID: 36851083; PMCID: PMC9962729.

3.Sun Z, Zhao Y, Wei Y, Ding X, Tan C, Wang C. Identification and validation of an anoikis-associated gene signature to predict clinical character, stemness, IDH mutation, and immune filtration in glioblastoma. Front Immunol. 2022 Aug 25;13:939523. doi: 10.3389/fimmu.2022.939523. PMID: 36091049; PMCID: PMC9452727.

4. Liu X, Nie L, Zhang Y, Yan Y, Wang C, Colic M, Olszewski K, Horbath A, Chen X, Lei G, Mao C, Wu S, Zhuang L, Poyurovsky MV, James You M, Hart T, Billadeau DD, Chen J, Gan B. Actin cytoskeleton vulnerability to disulfide stress mediates disulfidptosis. Nat Cell Biol. 2023 Mar;25(3):404-414. doi: 10.1038/s41556-023-01091-2. Epub 2023 Feb 6. PMID: 36747082; PMCID: PMC10027392.

Changes in the text: None.

At the signature construction level (##Identification of differentially expressed DRG signature), TCGA data were fitted after splitting the data into training and validation. RPN1, SLC2A1, SLC2A4, and SLC7A11 were found to be significant. The GEO data were not used at this step. The GEO datasets were only used (mentioned) as cross-referencing (Line 236), right?

Reply: Yes. To build prognostic models, the TCGA dataset was divided into a training set and a validation set. The GEO dataset was utilized in two distinct manners. Differential expression analysis was performed on the GEO dataset, integrating DRGs to identify those with differential expression. Additionally, an independent GEO dataset was employed for validation to verify the accuracy and generalizability of our model developed using the TCGA dataset. Both internal and external validation methods were used, further assessing the predictive capability of the prognostic model.

Changes in the text: None.

Given the four genes are a small subset of 24 DRG genes previously identified and AUCs (e.g., 0.794) reported on lines 277-279, it leaves a big room to improve, and as a result, a limitation analysis or statement of the study should be given.

Reply: Thank you very much for your advice. We add the limitation of this paper. (see Page 14, line 454-456)

Changes in the text: Fourth, the model we have developed is based on some DRGs genes, but the potential of other DRGs in liver cancer research remains to be investigated.

Minors:

In Section ## Real-time quantitative PCR (RT-qPCR), it's not clear to me what new experiments have been done. The authors used published data to conduct the research, so all work is computational, right? It would be helpful for review to mention which raw data to analyze. This issue appeared in several sections.

Reply: Our study comprises two components: analysis of public databases and experimental validation. Four genes were identified through bioinformatics screening, followed by experimental validation, primarily employing intracellular RT-qPCR to analyze expression differences between normal liver and liver cancer cell lines, thereby further corroborating the findings from public databases.

Changes in the text: None.

In Section ##Validation of the four DRGs, cell lines were compared. The same question as the above point.

Reply: At the cellular level, RT-qPCR was conducted on normal hepatocytes and HCC cell lines to confirm the expression differences of the four DRGs.

Changes in the text: None.

In Section ##Identification of differentially expressed DRG signature, three datasets were mentioned. I saw one dataset has only 6 samples (patients). How were these 6 samples used in the analysis?

Reply: I apologize for any confusion regarding our description. To clarify, the reference is to six genes, not the number of samples. Following the screening of 24 DRGs, six genes were

identified as differentially expressed. To develop a simplified prognostic model, univariate, LASSO, and multivariate analyses were employed to select these six genes, Subsequently, four of these genes were chosen for in-depth study.

Changes in the text: None.

As to high-risk and low-risk, discussions on gene signatures will be meaningful. This part seemed missed.

Reply: We appreciate your advice. The discussion section includes an analysis of gene signatures. (see Page 13, line 400-408)

Line 31: is this line a typo?

Reply: I am very sorry for your misunderstanding. This is a running title that has now been changed. we have modified our text as advised. (see Page 1, line 30)

Changes in the text: Running Title: Wang et al. DRGs and immune features in HCC.

Reviewer C

1. Reference

a. References 6 and 16 are the same. Please delete one of them and update the citations in the paper.

Reply: We have deleted reference 16 and updated reference.

b. The authors mentioned "studies...", while only one reference was cited. Change "Studies" to "A study" or add more citations. Please revise. Please number references consecutively in the order in which they are first mentioned in the text.

Previous studies have identified 24 DRGs (6).

Reply: We added 2 references and updated them.

Changes in the text: Previous studies have identified 24 DRGs (6, 12,13).

12. Feng Z, Zhao Q, Ding Y, et al. Identification a unique disulfidptosis classification regarding prognosis and immune landscapes in thyroid carcinoma and providing therapeutic strategies. Journal of cancer research and clinical oncology 2023;149(13):11157-70.↵
13. Xue W, Qiu K, Dong B, et al. Disulfidptosis-associated long non-coding RNA signature predicts the prognosis, tumor microenvironment, and immunotherapy and chemotherapy options in colon adenocarcinoma. Cancer cell international 2023;23(1):218.↵

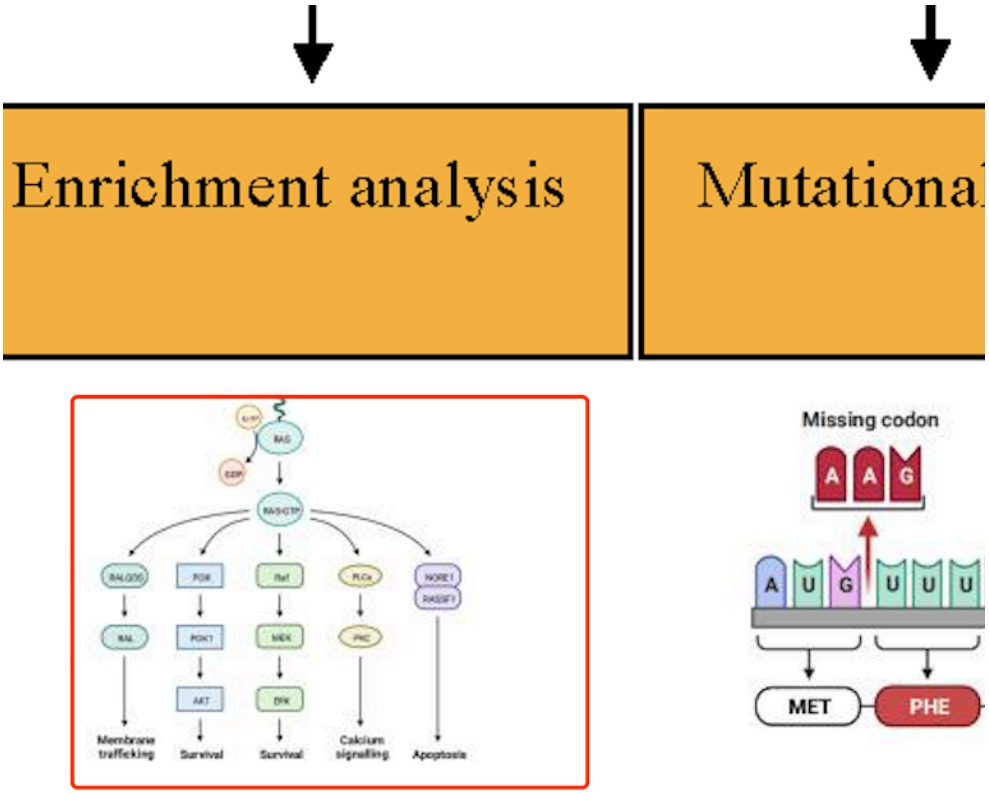
Studies have shown that the knockdown of RPN1 in vitro inhibits breast cancer cells proliferation, as well as induces cell apoptosis via endoplasmic reticulum stress (27).

Reply: Thank you very much for your advice. we have modified our text as advised. (see Page 13, line 396-398)

Changes in the text: A study have shown that the knockdown of RPN1 in vitro inhibits breast cancer cells proliferation, as well as induces cell apoptosis via endoplasmic reticulum stress (26).

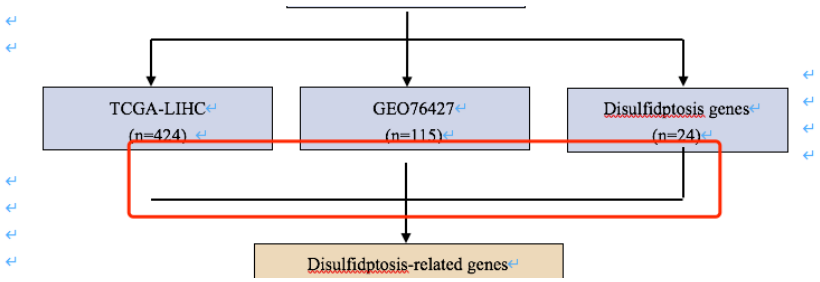
2. Figure 1

a. This image in figure 1 is not clear enough. It would be much appreciated if you could provide it with a higher resolution as possible as you could. The preferred format is JPG or TIFF.



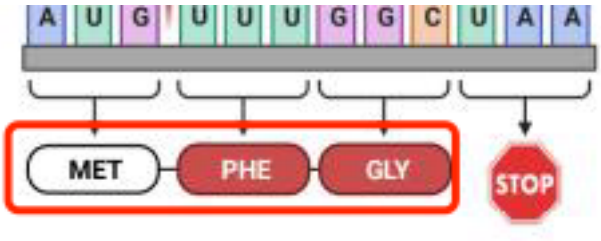
Reply: We have re-provided high quality images and rename it as “Figure 1-supplement ” and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript.

b. The framework is incomplete. Please revise.



Reply: Thank you very much for your advice. we have modified our figure 1 as advised and rename it as “Figure 1-revised” and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript.

c. Please indicate the full term of these abbreviations in the figure legend.



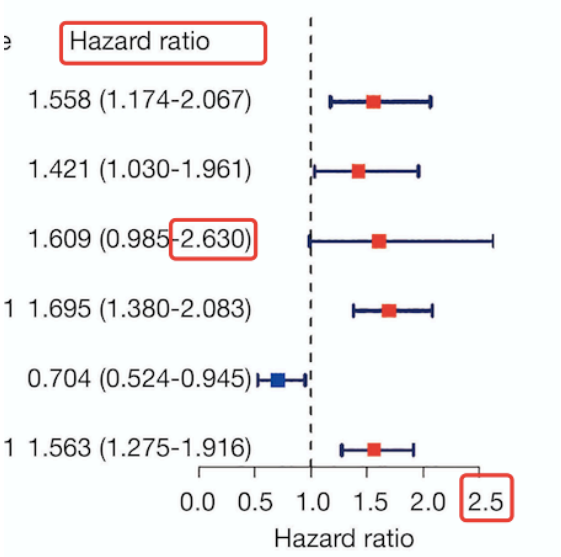
Reply: Thank you very much for your advice. we have modified our text as advised. (see Figure1 legend)

Changes in the text: MET, **Methionine; PHE, Phenylalanine; GLY, Glycine.**

3. Figure 2

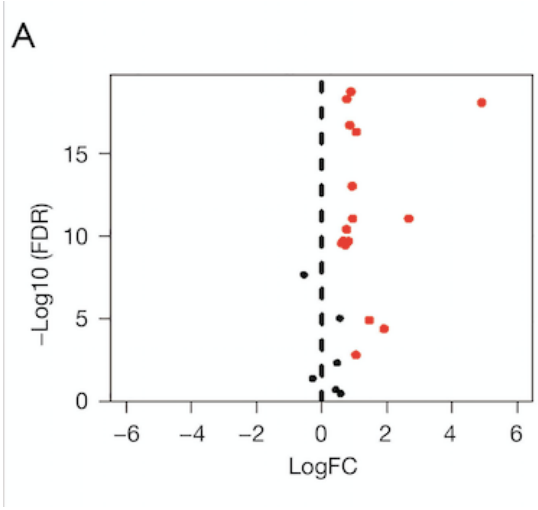
a. Please add (95% CI) after HR.

b. To standardize the results, the part that exceeds the horizontal coordinates should be indicated by arrows, or please extend the X-axis.



Reply: Thank you very much for your advice. we have modified our figure 2 as advised and rename it as "Figure 2-revised" and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript.

c. Please explain the meaning of black and red dots in the figure legend 2A.

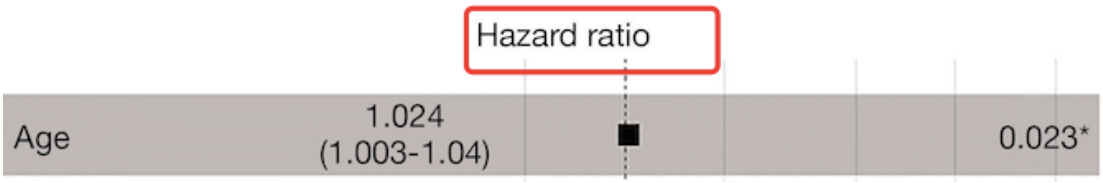


Reply: Thank you very much for your advice. we have modified our text as advised. (see Page 21, line 640-641)

Changes in the text: Black dots indicate genes with meaningless changes and red dots indicate genes with meaningful changes.

4. Figure 4

a. Please add (95% CI) after HR.



b. There are no symbols "**, ****" in figure 4, but they were explained in the figure legend. Please check and revise.

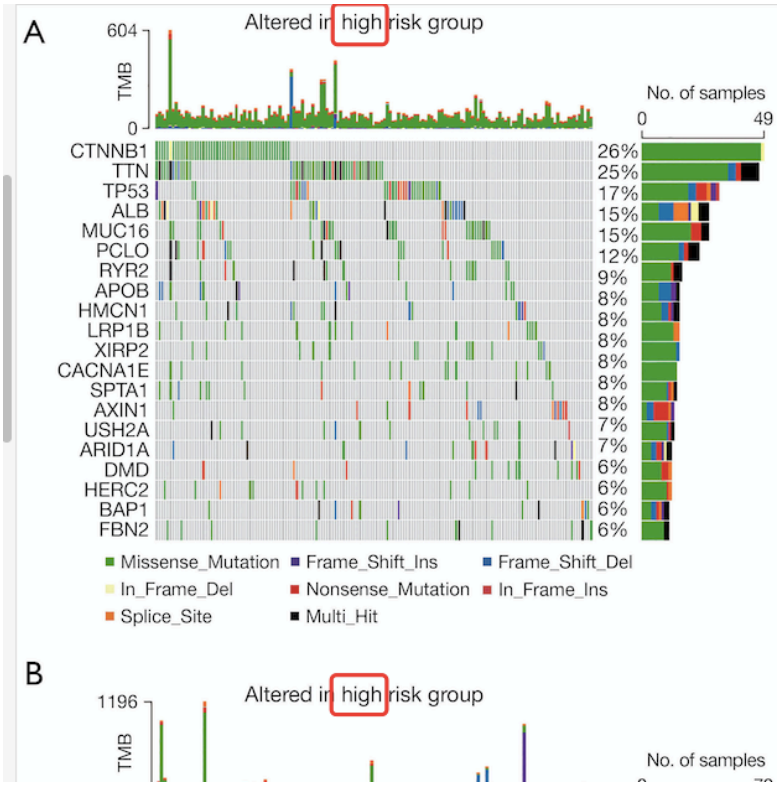
651 *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; ****, *P*<0.0001. HCC, hepatocellular carcinoma;
652 OS, overall survival; ROC, receiver operating characteristic; AUC, area under the curve.

Reply: Thank you very much for your advice. we have modified our figure 4 and its legend as advised and rename it as "Figure 4-revised" and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript. (see Page 24, line 671)

Changes in the text: *, *P*<0.05; ***, *P*<0.001.

5. Figure 6

Please check if one of them should be "low".



Reply: Thank you very much for your advice. we have modified our figure 6 as advised and rename it as "Figure 6-revised" and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript.

6. Figure 8 and Figure 11

Please indicate the meaning of 'ns' in figure legend.

Reply: Thank you very much for your advice. we have modified our text as advised. (see Page 28, line 707; see Page 31, line 727)

Changes in the text: Ns: no significance.

7. Figure 1

RT-qPCR or RT-PCR? Which one is correct? Please check and revise.

658 scRNA-seq, small conditional–sequencing; RT-PCR, real-time polymerase chain

659 reaction; MET, Methionine; PHE, Phenylalanine; GLY, Glycine.

Reply: RT-qPCR. we have modified our text as advised. (see Figure1 legend)

Changes in the text: RT-qPCR, real-time quantitative polymerase chain reaction.

8. Figure 4C

It seems that % on the x-axis and y-axis should be deleted. Please check and revise.

Reply: Thank you very much for your advice. we have modified our figure 4 as advised and rename it as “Figure 4-revised” and send the revised figure(s) as separate file(s) by email. And also replace the original one in the main manuscript.